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Award Number: DAMD17-01-1-0542

TITLE: BRCA2 is an Essential Component of the Rad51-dependent DNA Repair Complex

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REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Final (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
BRCA2 is an Essential Component of the Rad51-dependent DNA Repair Complex

5. FUNDING NUMBERS
DAMD17-01-1-0542

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The BRCA2 gene is associated with hereditary tendency to breast cancer. Mutations in BRCA2 cause a dominantly inherited predisposition to breast cancer. Recent evidence has indicated that BRCA2 protein plays a role in genome stability and in DNA repair mediated by homologous recombination. The BRCA2 protein has been shown to physically interact with Rad51, the key protein for DNA recombinational repair. In these BRCA2-deficient cells, formation of Rad51 foci is severely impaired. The evidence for the functions of BRCA2 in DNA repair has suggested a new concept for their role in predisposition to breast cancer. BRCA2 consists of two Rad51-binding domains, eight BRC repeats and a C-terminal region. These eight conserved BRC repeats (designated as BRC1 to BRC8), located in the central portion of the protein, are encoded by exon 11 and cover nearly a third of the protein. To demonstrate that BRCA2 is an essential component of the Rad51-dependent DNA repair complex and understand how BRCA2 regulates homologous recombinational repair (HRR), we have made construct of BRC1-4, BRC5-8 and BRC1-8 fragments of BRCA2 and to determine how it would affect the complex formation of Rad51 paralogs and *in vitro* biochemical activities of Rad51.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, BRCA2, DNA repair complex	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The BRCA2 gene is associated with hereditary tendency to breast cancer (reviewed in [1, 2]). Mutations in BRCA2 cause a dominantly inherited predisposition to breast cancer. Recent evidence has indicated that BRCA2 protein plays a role in genome stability and in DNA repair mediated by homologous recombination. The BRCA2 protein has been shown to physically interact with Rad51 [3-6], the key protein for DNA recombinational repair. Cells lacking BRCA2 function are hypersensitive to ionizing radiation and exhibit defective DNA repair [7-9]. In these BRCA2-deficient cells, formation of Rad51 foci is severely impaired. The evidence for the functions of BRCA2 in DNA repair has suggested a new concept for their role in predisposition to breast cancer.

The BRCA2 gene encodes a large protein of 3418 amino acids with a molecular weight of 384-kDa [10, 11]. This protein consists of two Rad51-binding domains, eight BRC repeats [3] and a C-terminal region [4, 5, 15]. These eight conserved BRC repeats (designated as BRC1 to BRC8) [12], located in the central portion of the protein, are encoded by exon 11 and cover nearly a third of the protein. Since the very large size of BRCA2 might hamper attempts to obtain the full-length protein, we therefore focus our efforts on the BRC repeats, which are the known functional domains for Rad51-binding of BRCA2. The domain constructs used in our study are listed in Figure 1. Our aim is to investigat how BRCA2 regulates homologous recombinational repair (HRR), mainly its effects on the complex formation of Rad51 paralogs and *in vitro* biochemical activities of Rad51 [16-18].

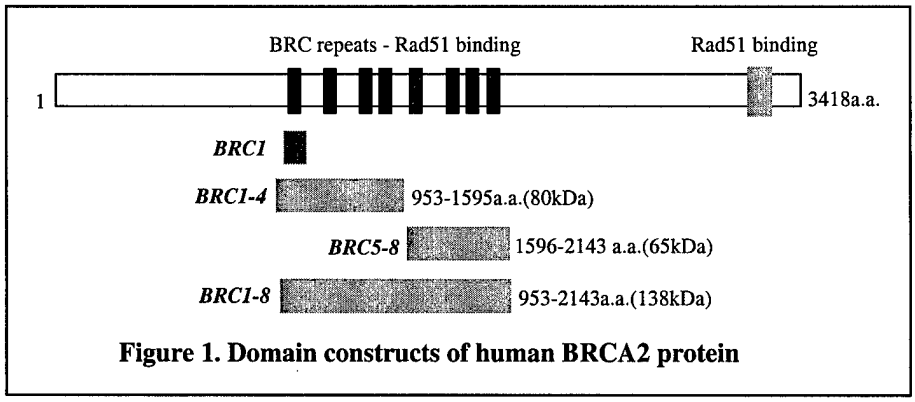

Figure 1. Domain constructs of human BRCA2 protein

Body

Specific aim 1. To determine whether BRCA2 or BRC repeats of BRCA2 protein forms a stable complex with Rad51 paralogs. Our results using a baculovirus co-expression system and Ni-NTA pull-down experiments have supported that Rad51 and five Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3) [19-25] interact simultaneously and form a novel complex. We propose to use these strategies to demonstrate that BRCA2 is an essential component in the Rad51-dependent recombinational complex, interacting with Rad51 to form a stable complex with Rad51 paralogs and to facilitate assembling of the complex formation.

Final results:

However, more recent evidence supported that the five Rad51 paralogs form four types of multiprotein complex in human cells, including Rad51B-Rad51C, Rad51D-XRCC2, XRCC3-Rad51C and Rad51B-

Rad51C-Rad51D-XRCC2 [26-28], instead of a single five-protein complex. Our *in vitro* evidence further showed the complex formation of Rad51-Rad51C-Rad51B (Figure 2). We therefore changed our aim to test whether BRCA2 forms a stable complex with Rad51-Rad51C-Rad51B. We would like to demonstrate that BRCA2 is an essential component in the Rad51-dependent DNA repair complex, interacting with Rad51 to form a stable complex with Rad51C-Rad51B and to facilitate assembling their complex formation. The Ni-NTA pull-down and gel filtration have been using to reach this goal.

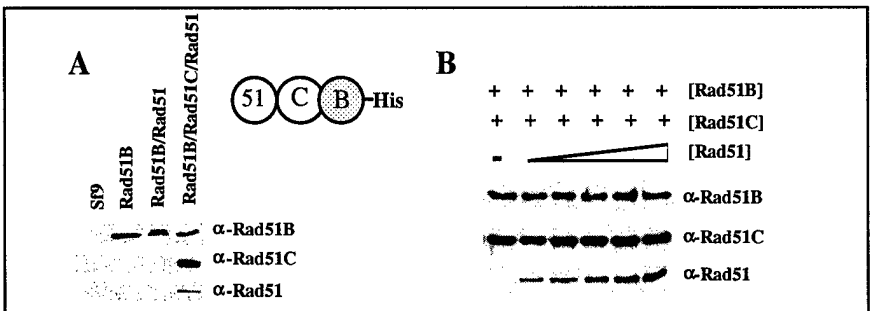

Figure 2. Complex formation of Rad51-Rad51C-Rad51B *in vitro*. A) Ni-NTA pull-down experiments using baculovirus co-expressed insect cells *lane 1*, uninfected Sf9 cell extract; *lane 2*, Sf9 singly infected with 6x(His)-tagged Rad51B; *lane 3*, Sf9 doubly co-infected; and *lane 4*, Sf9 triply co-infected. Each gel was run in triplicate, and three Western blots were done, probing with three different antibodies. **B) Ni-NTA pull-down experiments using purified Rad51, Rad51B and Rad51C proteins.** Rad51B and Rad51C were mixed (molar ratio = 1:1) with increasing amounts of Rad51 in the presence of 500μg/ml of ethidium bromide. The pull-down samples were subjected to Western blot analysis with Rad51, Rad51B or Rad51C antibody.

We have used the baculovirus expression system to co-express BRCA2 fragments with Rad51-Rad51C-Rad51B multiprotein compelx. The BRC1-4, BRC5-8 and BRC1-8 fragments have been PCR amplified using our BRCA2 full-length cDNA as a template, and individually constructed into a 6xHis-tagged baculoviral vector. We have already co-expressed the 6xHis-tagged BRC1-4 (or BRC1-8) domain with untagged Rad51, Rad51B and Rad51C in Sf9 cells. The expression of each protein was confirmed by Western Blotting using either α-BRC4 (aa1323-1346) or α-BRC5 (aa1651-1821) antibody and α-Rad51, α-Rad51B, and α-Rad51C antibody. The Ni-NTA magnetic beads will be used to pull down the 6xHis-tagged BRC fragments and whether Rad51, Rad51B and Rad51C simultaneously associates with the BRC proteins will be determined. A gel filtration column will further used to run the co-purified sample for determining the formation of a native protein complex.

Specific aim 2. To determine whether BRCA2 or BRC repeats of BRCA2 protein regulates the biochemical activities of Rad51. The direct interaction between BRCA2 and Rad51 has been demonstrated by immunoprecipitation and by a yeast two-hybrid assay [3-6]. However, it is not known how the interaction regulates homologous recombinational repair (HRR). Recent evidence [29] for BRC3 and BRC4 inhibition of nucleoprotein filament formation by Rad51 with DNA implies a role for BRCA2 in mediating the action of Rad51. We therefore propose to investigate the hypothesis that the BRCA2 protein functions to control the actions of Rad51 by directly modulating the biochemical activities of Rad51 — DNA binding, ATPase, and strand pairing and transfer — through binding to Rad51, and thereby consequently influences HRR. We have established an assay system for each of the biochemical activities of Rad51. Using these assays, the effects of BRCA2 on the biochemical activities of Rad51 will be examined.

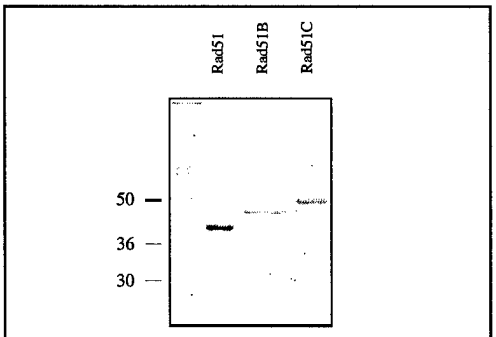

Figure 3. Purified Rad51, Rad51B and Rad51C proteins

Final results:

(1) **Cloning, expression and purification of Rad51 paralog baculoviral proteins.** We have expressed the human Rad51 protein and two Rad51 paralogs, Rad51B and Rad51C, in insect cells Sf9 using the baculovirus system. These three proteins were further individually purified to homogeneity using sequential column chromatography (Figure 3).

(2) **Cloning, expression and purification of the BRC fragments.** We have employed the baculovirus strategies to express the BRC fragments of BRCA2, including BRC1-4, BRC5-8, and BRC1-8 domains (Figure 4). They encode proteins of 80-kDa, 65-kDa, and 138-kDa, respectively. Each recombinant protein has been individually expressed in Sf9 cells (Figure 4) and the identity of the proteins was confirmed by Western Blotting using either α-BRC4 (aa1323-1346) or α-BRC5 (aa1651-1821) antibody (Figure 5) as well as α-His antibody. The BRC1-4 and BRC1-8 fragments have been further purified using a Ni-NTA column. Unfortunately, lots of proteolysis products were observed for both proteins after Ni-NTA purification. To solve the problem, we are currently co-expressed the individual fragment with Rad51 and hope that the interactions between BRCA2 and Rad51 will help stabilizing the BRCA2 structure and will make the co-purification of BRC1-4/Rad51 or BRC1-8/Rad51 complex possible.

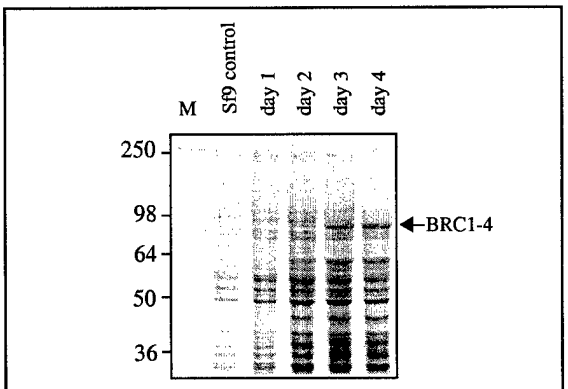

Figure 4. Baculovirus expression of BRC1-4 fragment of human BRCA2 protein

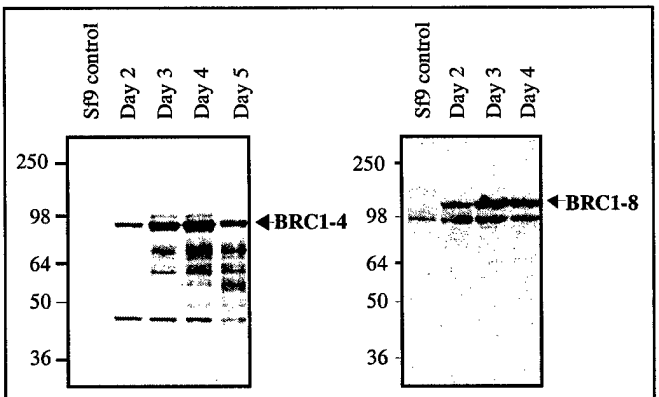

Figure 5. Baculovirus expression of BRC1-4 and BRC1-8

2.1. To determine whether the BRCA2 protein mediates the DNA binding activity of Rad51. The BRCA2 protein has been shown to contain multiple Rad51-binding sites [3-6, 15], suggesting that BRCA2 may bind several molecules of Rad51 simultaneously and serve as a scaffold for Rad51 assembly to DNA binding as filaments. The evidence indicates the possibility of DNA binding of Rad51 mediated by BRCA2. Therefore, it will be determined whether BRCA2 protein mediates the DNA binding activity of Rad51.

We have established a gel shift assay to determine the DNA binding activity using [^{32}P]-labeled oligonucleotides. Using this assay, we have examined the DNA binding activity of Rad51, Rad51B and Rad51C proteins (Figure 6). We have shown that Rad51B and

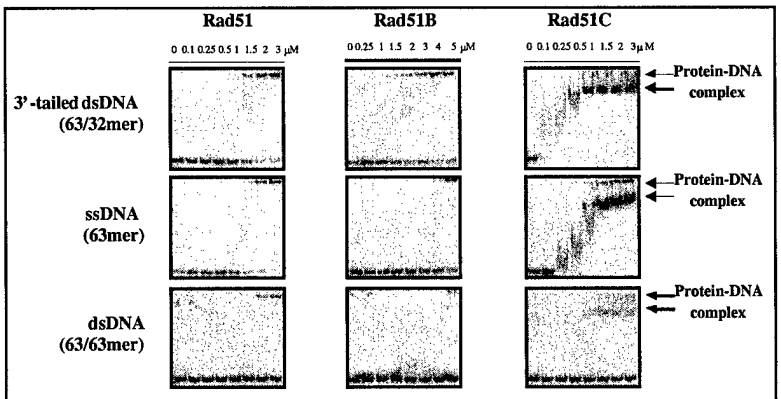

Figure 6. DNA binding activity of human Rad51, Rad51B and Rad51C

Rad51C bind both single-and double-stranded DNA (ssDNA and dsDNA), and show preference for tailed dsDNA. The effects of the BRC1-4, BRC5-8 and BRC1-8 fragments on the DNA binding activity of Rad51 will be determined using this assay.

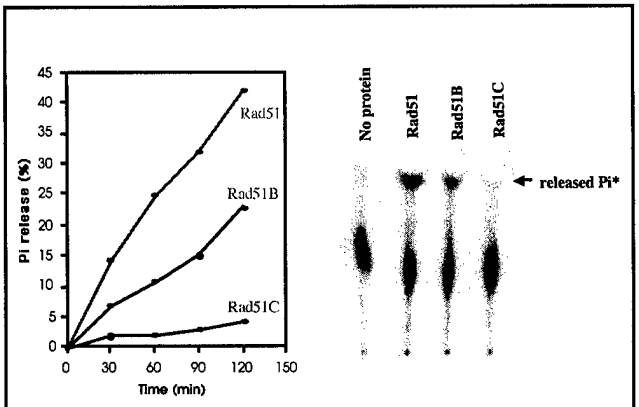

Figure 7. ATPase activity of Rad51, Rad51B and Rad51C in the presence ofssDNA

2.2. To determine whether the BRCA2 protein mediates the ATPase activity of Rad51. It has been shown that the BRCA2-binding region of human Rad51 (amino acids 98-339) is conserved in the *E. coli* homolog protein RecA. This region has been demonstrated to contain ATPase activity and is involved in oligomer formation and recombination. Therefore, whether BRCA2 functions to mediate the ATPase activity of Rad51 will be examined.

We have established an *in vitro* ATPase assay and determined the ATPase activity of the Rad51, Rad51B and Rad51C proteins (Figure 7). We have investigated the effect of the BRC1 domain of BRCA2 on the ATPase activity of Rad51. Various ratios of BRC1 and Rad51 were tested for the ATPase activity and an inhibitory effect was observed (Figure 8) — about 3.5 fold of inhibition was found with BRC1/Rad51 = 2. The suppression effect of the BRC1 domain on the ATPase activity of Rad51 suggests that the BRC1 domain of BRCA2 play a role in regulating the ATP binding and/or hydrolysis of Rad51.

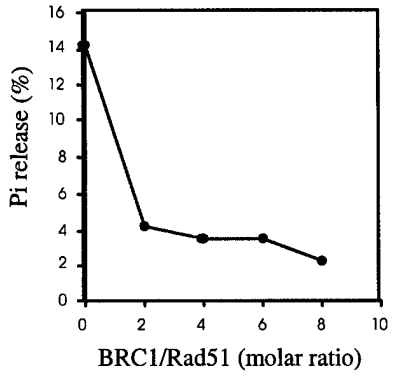

Figure 8. Inhibition of the ATPase activity of Rad51 by BRC1 peptide

2.3. To determine whether the BRCA2 protein mediates the homologous pairing and strand transfer activity of Rad51. The key biochemical activity of Rad51 in the recombinational DNA repair process is to promote strand pairing and exchange between two homologous DNA strands [16-18]. It is very likely that the interaction between BRCA2 and Rad51 influences the strand transfer activity of Rad51 and leads to effects on homologous recombination.

We have established a DNA strand transfer assay using ssDNA 63mers and [^{32}P]-labeled dsDNA 32/32mers as the substrates. The DNA strand transfer activity of Rad51 was determined as a

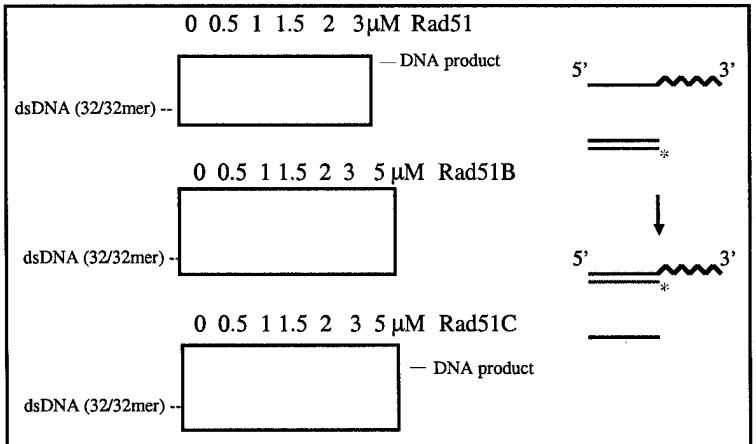

Figure 9. DNA strand transfer activity

control. The strand transfer products (3'-tailed 63/32mer) were observed and the amount of product formation is dependent on the concentration of Rad51. We also demonstrated that Rad51C displays DNA strand transfer (Figure 9) in an ATP-independent manner (data not shown), while Rad51B shows no such activity. The effects of BRC1-4, BRC5-8 and BRC1-8 on the DNA strand transfer activity of Rad51 will be examined.

Conclusions and summary

To demonstrate that BRCA2 is an essential component of the Rad51-dependent DNA repair complex and understand how BRCA2 regulates homologous recombinational repair (HRR), we have made the N-terminal 6xHis-tagged baculoviral construct of BRC1-4, BRC5-8 and BRC1-8 fragments of BRCA2. These three individual fragments have been successfully co-expressed with Rad51, Rad51B and Rad51C in insect cells. The Ni-NTA pull-down experiments are currently carried out to determine the complex formation between these proteins.

The BRC1-4, BRC5-8 and BRC1-8 proteins were also individually expressed in insect cells. The baculovirus titers were amplified, so that the expression level of proteins were elevated and were able to be detected by Coomassie staining. Each protein was purified using a Ni-NTA column. We observed lots of proteolysis product of each protein after Ni-NTA purification, indicating that the structure of the BRC repeats of BRCA2 protein is not stable and the protein tends to degrade. To solve the problem, each fragment was co-expressed with Rad51 individually. We hope to be able to co-purify a stable BRC1-4/Rad51, BRC5-8/Rad51 or BRC1-8/Rad51 complex for *in vitro* biochemical activity assays.

We have established three in vitro biochemical assays for Rad51 in our laboratory, including DNA binding, ATPase and DNA strand exchange. We have successfully used these three assay systems to determine the activity of Rad51B and Rad51C protein and the results have been prepared in manuscript for publication. We have examined the effect of BRC1 domain on the ATPase activity of Rad51 and found that the BRC1 domain inhibits this activity. The result suggests that the BRC1 domain of BRCA2 plays a role in regulating the ATPase activity of Rad51. We will apply these three assay approaches for determining the effects of the BRC fragments (BRC1-4, BRC5-8, BRC1-8) on the activity of Rad51 when we obtained the purified BRC/Rad51 protein complex. We have received an Idea Award from DOD to continue the follow up research.

Key Research Accomplishments

1. The *in vitro* complex formation of Rad51-Rad51C-Rad51B was demonstrated.
2. The BRC1-4 and BRC1-8 fragments of BRCA2 protein were individually co-expressed with Rad51, Rad51B and Rad51C in insect cells.
3. The BRC1-4 and BRC1-8 fragments of BRCA2 were expressed and purified. However, the whole fragments tend to become proleolysis during the course of purification, indicative of an unstable property of the protein.
4. The BRC1 domain of BRCA2 inhibited the ATPase activity of human Rad51, suggesting that the BRC1 domain of BRCA2 plays a role in regulating the ATP binding and/or hydrolysis of Rad51.

Reportable Outcomes

1. A manuscript related to this project "In vitro activities and complex formation of the human Rad51B and Rad51C DNA repair proteins" was in preparation and is close to submit for publication.
2. A postdoctoral fellow, Dr. Olga Miroshnychenko was hired for this study.
3. A DOD Idea Award was approved for funding to continue the study.

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